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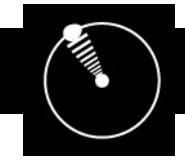
VIRUSES: CENTRIFUGATION



L. L. Bondoc Jr., BioPort Corporation, Lansing, MI, USA

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Viruses have proved to be detrimental as well as beneficial. They are notoriously infectious agents that are at the root of several major diseases in man, domesticated animals, and agricultural crops. However, their attenuated or noninfectious forms have been used as vaccines, enabling the development of immunity against particularly devastating diseases. Recently, replication-deficient viruses have been used as agents for gene delivery and as potential vaccine carriers, as they have evolved efficient mechanisms of infectivity.

Viruses are particulate in nature and are made up essentially of DNA or RNA, wrapped in a predominantly protein coat. They range in size from 20 to 2000 nm (0.02–2 μm) and in molecular weight from 4×10^6 to 2×10^9 Da. Many viruses possess an envelope that is typically derived from the host cellular membrane.

Initial isolation of viruses usually involves centrifugation, particularly density gradient centrifugation (DGC). For almost half a century DGC has been regarded as the most rapid, and reliable preparative procedure for the isolation of highly purified and concentrated virus preparations for subsequent physicochemical and biological characterization. As such, it is used as a benchmark against which alternative methods can be evaluated. To date the technique has permitted the isolation and subsequent characterization of a plethora of viruses belonging to at least 39 major families.

Centrifugal Separations

Although significant improvements in centrifugation hardware have led to increased operational efficiencies, the theory behind centrifugation and the variations of the technique as applied to viruses are well characterized. In a suspension of particles, the rate at which particles sediment when subjected to a centrifugal force depends on the nature of the particles, the nature of the medium, and the magnitude of the centrifugal force. For spherical particles, the sedimentation rate or velocity of the particle depends on a variety of factors as indicated in eqn [1], one of the many forms of the Svedberg equation:

$$dr/dt = [2r_p^2(\rho_p - \rho_m)\omega^2 r]/9\eta \qquad [1]$$

where dr/dt is the velocity of the particle; r_p is the radius of the spherical particle; ρ_p is the density of the particle; ρ_m is the density of the medium; ω is the angular velocity; r is the radial distance of the particle from the axis of rotation; the product $\omega^2 r$ is proportional to the centrifugal force; and η is the viscosity of the medium. It is possible to define a particle in terms of its behaviour in a centrifugal field by manipulation of eqn [1] to yield a simplified version of the Svedberg equation (eqn [2]) that uses the sedimentation coefficient, s, where:

$$s = (dr/dt)/\omega^2 r \qquad [2]$$

For most biological macromolecules, the magnitude of s is about 10^{-13} s, so this value is used as the unit of sedimentation, the Svedberg (S). The sedimentation coefficient for viruses varies between 40 and 4500 S, while for globular proteins it is 2–5 S.

Types of Separations

For a particular viral preparation, the most effective centrifugal separation procedure is one that yields a concentrate with significant recovery of bioactivity

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and high quality based on several measures of purity. There are three types of centrifugal separations available for viruses: (1) differential centrifugation; (2) rate-zonal centrifugation; and (3) DGC or isopycnic centrifugation. Differential centrifugation separates particles according to size as well as density (from eqn [1]), since denser particles will form pellets at a faster rate than less dense particles of the same mass. By choosing an appropriate centrifugal force and centrifugation time, it is possible to clarify a viral suspension from contaminating fermentation debris by first pelleting the contaminants at a given *g* force and leaving the virus in suspension, then pelleting the virus at a higher *g* force. Viruses that are unstable when pelleted can be sedimented on a cushion or plug of material (e.g. caesium chloride, sucrose, potassium tartrate, Nycodenz (Nyegaard & Co.), and glycerol) that has a density higher than that of the viral particles. The major problems with this mode of separation are the low yields and low resolution from contaminants. Differential pelleting is often used for the initial processing of heterogeneous mixtures, to obtain fractions that are enriched in the virus particles of interest prior to further purification.

In rate-zonal centrifugation particles move at different rates depending upon their mass. To avoid the co-sedimentation of particles of different sizes, samples are typically layered as a narrow zone on top of a density gradient. The gradient is used to facilitate the layering of the sample and to minimize convection currents in the liquid column during centrifugation that would otherwise disrupt the particle zones as they move down the tube. Rate-zonal separations are ideal for particles of uniform size but not for particles of the same type that are heterogeneous in size. Furthermore, even though separation conditions can be optimized, it is not yet possible to recover the separated fractions as fractionated species. For viral preparations this mode of centrifugation is primarily used for characterizations, such as molecular weight determination and the determination of possible interactions with other molecules.

The most common method of centrifugal separation for viruses is DGC, or isopycnic centrifugation. In this process the particles move until their density is the same as that of the surrounding medium. The particles are separated purely on the basis of their density, and their size only affects the rate at which they reach their isopycnic positions. Because the separation is an equilibrium process, run times are generally much longer than for rate-zonal or differential centrifugation. Prolonged centrifugation does not affect the separation as long as the gradient remains stable and the activity and integrity of the particles are not adversely affected by centrifugation. Materials are typically spun at 25 000–200 000 *g* for up to 20 h. Samples are loaded either on a pre-formed gradient or on self-forming gradient media.

Centrifugation Media

For isopycnic separations, the choice of media is important. There are several desirable characteristics for a medium, the most important being that the maximum density of the gradient is greater than that of the particles to be separated. In general viruses have buoyant densities in the range 1.1–1.5 g cm^{-3}. However, as a result of different levels of hydration of viral particles in different media, the densities of the particles can vary depending on the medium being used. The physico-chemical properties of the solutions of the gradient medium should be known, and it should be possible to determine the precise concentration of the medium using one or more of these properties (e.g. refractive index or densitometry). The medium should be inert and safe to use, and should not interfere with monitoring of the zones of fractionated material within the gradient (e.g. by ultraviolet or visible absorbance, radioactivity counting, protein determination, etc.). It should be easy to separate the sample material from the gradient medium (by dialysis, ultrafiltration, or centrifugation) without loss of the sample or sample activity. Ideally the medium should also form solutions of low ionic strength with low viscosity and be iso-osmotic with the virus.

Gradient media for DGC are either ionic or nonionic. Commonly used ionic media include caesium salts (e.g. caesium chloride), potassium salts, rubidium salts and sodium salts (e.g. sodium chloride). These materials are used to form solutions with maximum buoyant densities of 1.4–2.6 g mL^{-1}. Gradients of caesium salts, especially caesium chloride, are used almost exclusively for virus purification. They can be pre-formed using any of the standard techniques or they can be formed *in situ* by centrifugation. Solutions containing caesium salts are highly ionic, and while they are nonviscous, they all have high osmolarities. Gradients formed from these salts, differ with respect to their solubility, maximum density, activity and steepness, all of which can affect the banding of materials.

Nonionic gradient media can be subdivided into carbohydrates, iodinated gradient solutes, colloidal silica suspensions and proteins. Sucrose, a disaccharide, has been widely used for the isopycnic fractionation of viruses. Its popularity is due to its inertness towards biological materials, ready availability, low cost, and stability. The main disadvantages of sucrose

include its high osmotic strength, high viscosity, hypertonicity for solutions more concentrated than 9% (w/v) and rather low buoyant density of 1.03 g mL^{-1}. Sucrose gradients must be pre-formed for isopycnic fractionations.

To circumvent the problems that arise from fractionating osmotically sensitive particles in high osmotic strength sucrose solutions, several polysaccharides have been used as gradient media. These include glycogen, dextrans, and Ficoll (Pharmacia). Ficoll is produced by the chemical copolymerization of sucrose molecules with epichlorohydrin to give a polymer with a molecular weight of 400 kDa. Ficoll solutions below 20% (w/v), equivalent to a buoyant density of 1.07 g cm^{-3}, have a relatively low osmolarity, although at higher concentrations the osmolarity rises sharply. Gradients of Ficoll, which have a higher viscosity and better stability than sucrose gradients, must be prepared using a gradient mixer.

Most iodinated gradient media used in the separation of viruses are derivatives of triiodobenzoic acid to which hydrophilic groups have been attached to increase water solubility. The ionic forms of these compounds include the sodium or *N*-methylglucamine salts of metrizoate, diatrizoate, and iothalamate, and the nonionic forms include metrizamide and Nycodenz. These materials form stable solutions at buoyant densities up to 1.45 g cm^{-3}. Iodinated compounds have several advantages, including much lower osmolarities and viscosities than sucrose at all densities. Gradients of these media can be preformed or generated in place.

Colloidal silica gradients have been used for several years, but only one preparation, namely Percoll (Pharmacia), has been developed for centrifugation. In this particular preparation the silica particles are coated with polyvinylpyrrolidone, which minimizes their interaction with biological material and also stabilizes the colloid against freezing and thawing and the presence of salts. Its solutions are isoosmotic and its low viscosity facilitates the rapid banding of viruses. However, Percoll is precipitated at low pH and solutions of high ionic strength destabilize the colloidal suspension. Gradients of Percoll readily self-form, or can be pre-formed using a simple mixer. Percoll forms suspensions at buoyant densities up to 1.13 g cm^{-3}. The removal of Percoll from virus solutions can be problematic because Percoll particles (17–30 nm in diameter) are very close in size of some viruses.

Proteins have a hydrated buoyant density of approximately 1.27 g cm^{-3} and can be used as gradient media, but no applications to viruses have been reported.

Types of Gradients

Pre-formed gradients for DGC of viruses can be continuous or discontinuous. Continuous gradients may be linear, convex, or concave and are usually prepared using a dedicated gradient former. Discontinuous or step gradients are prepared by successively layering solutions of different density. Pre-formed gradients must be handled very carefully prior to centrifugation to avoid gradient disruptions caused by vibration or temperature variations. The virus sample itself, which must have a density less than that of the top of the gradient, is gently layered onto the gradient before centrifugation is started. To minimize changes in the density profile at the top of the gradient, the sample volume should be small compared with the gradient volume.

For self-forming gradients the initial sample volume is not a concern, as the sample is either mixed with a concentrated solution of the gradient solute or solid gradient solute is added to give the correct initial density. The duration of centrifugation for self-forming gradients is longer than that for pre-formed gradients since time is required to form the gradient.

Rotors

DGC can be carried out in all the available types of rotors. Preparative centrifuge rotors are classified into four main types, namely swing-out (swinging bucket), fixed angle, vertical, and zonal. In swing-out or horizontal rotors, the tubes of sample solutions are placed in individual buckets that move out perpendicular to the axis of rotation as the rotor rotates. This creates a long migration path to separate viruses along the density gradient and requires a long period to achieve significant separation. Horizontal rotors can be spun to attain maximum speeds corresponding to 100 000 *g* or more.

In fixed angle rotors the tubes are at a fixed angle (varying from 14° to 40°) to the axis of rotation, and when the rotor rotates the solution reorients in the tubes. This reorientation enhances the loading capacity of the isopycnic gradients. Rotors with shallow angles are more efficient at pelleting because the sedimentation pathlength is shorter. Fixed angle rotors are designed to operate up to very high centrifugal forces (>600 000 *g*).

As the name suggests, in vertical rotors the tubes are held in a vertical position, and centrifugal forces similar to those for fixed angle rotors can be achieved. When the vertical rotor turns, the solution begins to reorient through 90°. Vertical rotors thus have short sedimentation pathlengths, so the diameter of the

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tube and the capacities of the gradients in these rotors are higher than in horizontal and fixed angle rotors.

Zonal rotors are often used for gradient separation. Although the sample is pumped into a hollow rotor chamber, the working principle of these rotors is similar to that for vertical rotors, as it is the gradient solution that reorients during the run, before the sample is introduced under centrifugation. There are two types of zonal rotors, namely batch and continuous flow, that differ based on the volume of sample they can be used to process. Batch type centrifugation is typically used for 10–200 mL samples while for larger sample volumes of 100 L or more, continuous flow centrifugation is required. For volumes 200 mL to 100 L, vertical rotors can be used.

Recovery

Recovery of viral particles from DGC is performed either manually or automatically. After centrifugation density gradients can be recovered or unloaded from the bottom, middle or the top of the tube. The methods for unloading gradients from the bottom of the tube include bottom puncture of the tube or withdrawal using a narrow tube inserted through the gradient to the bottom. Targeted bands from within the gradient can also be unloaded by puncturing the tube at the appropriate position. The methods for unloading gradients from the top of the tube include direct unloading from the top or collection by upward displacement of the gradient by introducing a dense, preferably immiscible liquid at the bottom of the centrifuge tube. Automated collection systems with flow-arrest or volumetric monitoring are available commercially. Automated recovery systems require a heavy displacement solution such as 65% sucrose or Maxidens (Nyegaard & Co.), an inert, nonviscous organic liquid immiscible with aqueous gradients. Great care must be taken in fractionating gradients after centrifugation since resolution is easily lost at this stage. All operations sh o uld be designed to minimize disturbance of the gradient.

Conclusion and Future Developments

DGC is still the method of choice for the initial, relatively quick isolation of novel viral particles. The gradient medium, gradient shape, type of rotor, and mode of recovery are determined empirically, for a particular viral preparation, to yield the highest possible recovery of bioactive purified virus and permit subsequent characterization and use.

The advent of gene therapy using viruses for gene delivery or as vaccine carriers has encouraged the development of scaleable procedures for virus isolation and purification based on centrifugation. With this impetus gradient media, rotor designs, and modes of recovery continue to be improved.

See also: **II/Centrifugation:** Theory of Centrifugation.

Further Reading

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